SEED SOAKING STUDIES

INTRODUCTION AND REVIEW OF LITERATURE

The seed occupies an unique position in the plant science. They are familiar but mysterious, as most of the known physiological processes associated with growth and development are concentrated in this small unit of mystery. In biological words, seed is a culmination of a phase of plant development which starts with fertilization and ends with desiccation.

All the viable seeds possess the ability to germinate if placed under suitable conditions necessary for germination. We will regard seed germination as that series of steps normally occurring prior to the emergence of the radicle from the seedcoat, or it is the transformation of seeds into seedlings. Among the plant phases seed germination and early seedling growth are considered critical for raising a successful crop as these indirectly determine the crop stand, density and consequently the yield of a resultant crop (Gelmond, 1978).

Both the external and internal environment profoundly influence the process of seed germination. Seeds of certain plants germinate immediately after they are harvested, in others they fail to germinate for sometime even if placed under suitable condition that are ordinarily favourable for germination, either due to some internal factor or due to specific requirements.

Germination is the resumption of metabolic activity and growth by the seed tissues, involving rehydration, utilization of nutrient reserves and the gradual development of synthetic systems which enable the young plant to assume an autotrophic existence (Streel and Opik, 1970). Optimum temperature, adequate moisture (water) level in the medium and the presence of oxygen in the balanced proportion in the atmosphere are the essential conditions which must be fulfilled for proper initial germination. Pulses in India are widely grown by small farmers in the semi-arid tropics or in rain-fed areas as a backyard subsistence crop. So, they need special care which will enable the resulting plants to resist overheating and dehydration, endure drought better, and yield higher under arid conditions.
Presoaking treatment of seeds is now considered to be an effective way for the physiological and biochemical manipulation of the plant growth right from seed germination to crop production. Presoaking hardening of seeds may be described as a treatment preliminary to germination during which seeds are moistened and dried back to original weight as designed by Henckel (1964), this is to activate certain physiological processes in the germinating seed.

The soaking environment also has profound effect on germination behaviour, which is brought about by following: (i) soaking volumes, (ii) soaking duration, (iii) soaking temperature, (iv) type and concentration of chemicals or PGRs used for soaking, (v) the variety of seed, its chemical composition and moisture content during soaking also affect the soaking behaviour. Seeds are sensitive to the volume of aqueous medium during soaking. Their germination is arrested by the presence of excess water in the substrate which is responsible for damaging the integrity of seed-coat and by interfering with its oxygen diffusion phenomena (Mehra, 1990; Malhotra, 1990; Pandya, 1992). In some plants, the positive effects of presoaking and drying were not noticed at all or while germination was hastened, the growth of the seedling was depressed (Parrish and Leopold, 1977; Powell and Matthews, 1978; Me Kersie and Tomes, 1980). The reason may be the prolonged soaking or soaking injuries due to lack of perfect standardization for optimum volume, temperature and duration of soaking. Prolonged soaking has been found to cause injury to seeds of many species; the injury appearing after quite different duration of soaking in different types of seeds (Rocs and Pollock, 1971; Heydecker, 1973; Saxena, 1985; Chowdhuri and Choudhuri, 1987). Heydecker (1967) cautioned that the initial process must be carried out under strictly controlled and hygienic conditions in order to avoid failures. The temperature of dehydration of seeds after soaking must not exceed 20°C or should be low (Roberts, 1948; Crocker and Barton, 1957). Similarly hydration of seeds at low (24-26°C) temperature and for limited duration and then dehydration also shows better germination performance (Mayer, 1974; Saxena, 1974).

In past most research on presoaking enhancement of germination has been carried out on the dryland grass species by a group of USDA researchers at Longan, Utah. The positive effect of
pre-treatments of seeds of many crop with various growth regulators and chemicals have been well reviewed (Chinoy et al., 1970; Saxena, 1974; 1989; Dasgupta and Basu, 1975; Basu, 1976; and Saxena and Pakeeraiah, 1986). The introduction of chemical growth regulators has added a new dimension to the possibility of modifying the plant growth (Mehrotra et al., 1983).

Presoaking treatments with PGRs, some phenolic salts, antipathogenic compounds and antioxidants are used to modify the crop by altering its germination and growth pattern of seedlings. The beneficial effects may be because of physiological and biochemical advancement of germinating seeds by triggering out some of the initial steps of germination or due to leaching of toxic metabolites from the seeds which leads to better germinability. All the beneficial effects are not induced to same degree in all the seeds. Different seeds behave differently with respect to number of pretreatment cycles, percentage moisture content, duration and volume of soaking and these in turn, affect greatly the physiological performance of the pretreated seeds as compared to untreated control seeds (Saxena, 1981).

Seed pretreatment is claimed to be beneficial when it is considered with much caution and with a newer perspective. Few fundamental points to be remembered during pretreatments are as follows: (i) the type of seeds to which it is applied, (ii) uniformity of seed, (iii) reduced chemical variability of seeds, (iv) type and concentration of PGRs applied, this implies both correct chemical with correct dosage rates at correct time of application, (v) uniform application throughout the treatment, (vi) optimum temperature, volume and duration of treatment, (vii) after treatment the degree and temperature of seed drying which gives better results.

In biochemical studies, three successive phases can be distinguished during germination of all seeds (Evenari, 1957; Katoh and Esashi, 1975; Lewak et al., 1975). The imaginary distinction of the three phases is based upon recognition of processes dominating during a given period of germination.

**First phase:** The colloids become hydrated and their structure rearranged, several enzymes regains their activity, diffusion of solutes into seeds, imbibition, rearrangement of cell membrane.
Second phase: True restoration of life in seed, dominant processes are hydrolysis and degradation of food reserves, synthesis of some hydrolytic enzymes.

Third phase: Accessibility of low molecular products coming from storage material. Synthesis of cell wall materials, proteins and RNA.

During biochemical studies of germination much of the work has been carried out on entire seeds, partly because of the practical difficulties involved in separating the embryonic axis from endosperm or cotyledon, which shows a very complex interaction between the two. Interactions are probably an integral part of the control germination. Separation into different parts obscures such interaction and gives clear picture of biochemical changes during germination, because the seedling has been a very good source of enzymes and other plant constituents but the contribution of the cotyledons to seedling development differ greatly in different plants, depending partly on type and size of cotyledon (Mayer, 1974).

Exogenous application of PGRs and different chemicals plays a major role in controlling the biochemical changes accompanying seed germination and seedling growth (Chinoy and Saxena, 1971; Takayanagi and Harrington, 1971; Younis et al., 1971; Mayer, 1974; Pandya, 1992; Thanki, 1993). However, results are inconclusive for many reasons, it is likely that hydrolytic processes begin during presoaking and the resulting simple sugars can be immediately utilized for synthesis upon germination, conversely, soaking seeds can be detrimental to germinability and should not be used unless definitely needed. An increase in protein content of pea seeds by soaking the seeds with IAA, NAA, GA, MH and 2-4D was also reported by Rao (1979). Positive effect of GA₃ on amylase activity of germinating seedling of Pennisetum typhoides (Huber and Sankhla, 1974), on pea cotyledon (Locker and Ilan, 1975), have been reported. The onset of proteolytic activity seems to be controlled in some cases by hormones action, by cytokinin in squash cotyledon (Penner and Ashton, 1966), in soyabean axis and cotyledon (Maheswari, 1987). Various proteolytic enzymes have been reported in germinating seeds of many cereals but onset of it’s activity differ in different seed types and till today little attention has been paid to real metabolic changes and enzyme activity in the pulse seeds during early hours of germina-
tion in relation to PGRs application.

Present knowledge of pretreatment or presoaking with various PGRs is now advocated to be a successful way to incorporate them into the seeds; which, in turn, exert their beneficial effects on physiological and biochemical processes of seed germination. Hence studies on presoaking various pulse seeds in water and in various PGRs at different concentrations were conducted to observe their performances on physiological and biochemical changes during seed germination.
2.A STANDARDIZATION STUDIES:

The experiment was carried out to standardize the requirement of optimum soaking volume, duration and temperature for four different pulse crops viz. mungbean [green gram, Vigna radiata (L) wilczck cv. K-851], urdbean [black gram, Vigna mungo (L) Hepper cv. T-9], cowpea (Vigna ungiuculata (L.) walp. cv. Pusa phalguni) and Pigeonpea [Cajanus cajan (L) Millsp. cv. BDN-2]. The certified seeds of all the four crops were used throughout experimentation and were graded carefully and stored at room temperature.

i) Seed soaking volume, duration and temperature:

Morphologically uniform seeds of mungbean, urdbean, cowpea and pigeonpea were weighed and soaked in distilled water in the following weight/volume (g/ml) ratio - 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10 for different duration (hour) 2, 3, 4, 5, 6, 8 and 10 hour (h). There after, the water soaked seeds were surface dried with blotting paper and finally dried to their original weight in a current of cool air. The control seeds were not soaked but dried along with the treated seeds (Basu and Dasgupta, 1974). After drying the seeds were kept in dessicators over fused calcium chloride for one week for moisture equilibrium and then germination performance was studied.

In second set of experiment the best suitable studied soaking volume and duration were kept constant in each crop and seeds were soaked at different temperature, i.e. room temperature (RT) = 32°C, BoD = 26±2°C and Fridge = 7°C and they were finally dried back to original weight as mentioned ab low water content in air-dried seeds metabolic activity is practically at a stand still (Street, 1966) with imbibition thove. The seed germination and seedling growth was studied in each type of seed on the basis of number of normal seedling (ISTA, 1985a, 1985b).

Germination and Seedling growth:

Treated seeds of mungbean, urdbean and cowpea were germinated by paper towel method at 26±2°C and under continuous white fluorescent light in BOD-incubator. The paper towel method was employed for above seeds because the seedling size increases very fast so to avoid
drying of uprooted seedlings during germination, as paper towel gives sufficient moisture. Seedling development assessed by these methods gave (i) rapid emergence due to sufficient moisture in contrast to petridishes where water uptake is restricted, (ii) more uniform germination, (iii) straight seedling growth, more seeds for each lot can be accommodated at a time.

In case of pigeonpea above method of germination gave repeated fungal contamination, this may be due to exposed seeds and excess moisture. So, here seeds were germinated in sterilised petridishes lined with filter paper (monol-X no. 1). Seeds were moistened with distilled water (DW) daily and petridishes were kept in BoD incubator for 10 days germination instead of 7 days as in other three crops because here growth and seedling differentiation is very slow.

Percent germination in each experiment were recorded by simple counting, seedling length (root length + shoot length, fresh and dry weight of each seedling part were recorded after 7 days in mungbean, urdbean, cowpea and after 10 days in pigeonpea. The experiment was replicated thrice and in each case 10 uniform seedling were selected to collect the data.

ii) Chemical Concentration:

Various PGRs were administered by presoaking treatment in concentration ranging from $10^{-4}$ molar (M) to $10^{-7}$M to the seeds.

Uniform sized, weighed seeds of mungbean and urdbean were soaked in double the volume (1 g in 2 ml) whereas cowpea and pigeonpea in six time (1 g in 6 ml) volume of distilled water (DW) and aqueous solution of different concentration ($10^{-4}$M to $10^{-7}$M) of PGRs viz. gibberellic acid (GA$_3$), Kinetin (KIN), naphthylacetic acid (NAA), Ethrel (ETH), indolebutyric acid (IBA) and abscisic acid (ABA). The soaking hours for mung bean and cowpea is 4 hours, urdbean 5 hours and for pigeonpea 6 hours. The untreated (UN) seeds served as the control (CON).

The treated seeds were moisture equilibrated and germinated as described earlier in 2.A(i).

Germination and seedling growth: as described earlier in 2.A(i).

Root, Shoot and leaf fresh weight: Uniformly germinated seedling were selected, surface dried with blotting paper and fresh weight of organ were recorded using single pan balance.
Seedling dry weight: Different organs (shoot, root and leaf) of surface dried seedling were kept at 80°C in air-oven till attainment of constant dry weight.

iii) In this set of experiment the seeds of all four crop were soaked in optimum volume of best concentration of each PGRs separately for optimum duration at optimum temperature. Then a part of seeds were not dried but freshly used for germination test and seedling performance study and other part of seeds were dried back to original weight for same germination performance studies.

2.B BIOCHEMICAL STUDIES:

Uniform seeds of mungbean [Vigna radiata (L) Wilczeck cv. K-851], urdbean [Vigna mungo (L) Hepper cv. T-9], cowpea [Vigna unguiiculata (L.) walp cv. Pusa phalguni], pigeonpea (Cajanus cajan cv. BDN-2) were subjected to PGRs pretreatment viz. Gibberellic acid (GA) 10^{-5}M, Kinetin (KIN) 10^{-6}M, naphthyl acetic acid (NAA) 10^{-6}M, Ethrel (ETH) 10^{-7}M, Indole-3-butyric acid (IBA) 10^{-6}M, abscisic acid (ABA) 10^{-6}M and distilled water (DW). Volume and duration of soaking in mungbean were 1:2 (w/v) for 4 hours (h), urdbean 1:2 (w/v) for 5 h, cowpea 1:6 (w/v) for 4 h and for pigeonpea 1:6 (w/v) for 6 h soaking. After that seeds were surface dried on blotting papers and finally dried to their original weight in a current of cool air. The untreated control seeds (UN) were not soaked but were dried similarly along with other seeds. Seeds, after initial drying, were kept in the desiccators over fused CaCl₂ for seven days for moisture equilibration. These seeds were germinated in petriplates for 24, 48, 72 and 96 h duration in BOD (26±2°C) incubators and were used for measurement of enzyme activity and metabolic changes during germination. In pigeonpea measurement was started after 48h of germination due to its slow growth.

i) Enzyme Activities:

Preparation of Enzyme Homogenate:

As illustrated in flow-chart I.
FLOW CHART I DEPICTING SEQUENCE OF ENZYME EXTRACTION

Embryo axis and Cotyledon  
Weighed separately and homogenised  
in 5 ml chilled acetone, left overnight at 4°C,  
Centrifuged at 10000 g for 15 min. at 4°C  

Residue  
Supernatant (discarded)  
Dissolved in 5 ml PO₄ buffer  
(0.02M, pH 6.4)  

Centrifuged at  
1000 g for  
15 min at 4°C  

Pellet resuspended  
in PO₄ buffer as (100 mg/10 ml)  
(0.02M, pH 6.4)  

Centrifuged  

Supernatant used for enzymatic analysis  
1) Amylase  
2) Protease  
3) Invertase  
4) Peroxidase  
5) Enzyme protein
Amylase activity:
Total amylase activity was determined by the method of Paleg et al (1962). To 1 ml of enzyme aliquote (illustrated in flow chart I), 1 ml solution of 10 mM CaCl₂ + .2 M NaCl was added for activity of amylase enzyme and to it 1 ml citrate buffer pH 5.0 (0.1M) and 5 ml of 0.1% starch was added and kept at 30°C for 30 min. To the above reaction mixture 1 ml I₂KI solution was added at 30°C and kept for 5 min, then volume was made to 20 ml by distilled water and OD was taken at 600 nm spectrophotometer. In control 1 ml DW in place of enzyme aliquot was added and for blank 1 drop of .1N sodium thiosulphate in the reaction mixture was used as blank. The activity of total amylase was expressed as mg starch hydrolysed/g fresh weight/30 min. The regression formula for calculation is X = 0.000345 Y - 0.023

Protease:
Protease activity was assayed following the method of Penner and Ashton (1967) as modified by Cruj et al (1970); the modification removes protease inhibitors.
1.0 ml of aliquot was mixed with 3.0 ml of phosphate buffer (0.2M, pH 7.0) and 2.0 ml of 0.5% casein solution (pH 7.0). The assay system was then incubated at 30°C for 1 h and then terminated by the addition of 2 ml of 15% (w/v) trichloroacetic acid (TCA) for 2 ml of reaction mixture and allowed to stand for 20 min. The tubes were centrifuged and supernatant free amino acids were estimated (using tyrosine as a standard) following the colorimetric method of Lowry et al (1951).
A suitable aliquot of supernatant (1.0 ml) was diluted to 2.0 ml with the DW and mixed with 4.0 ml of 12.5% (w/v) Na₂CO₃ and 1.0 ml of 0.1% CuSO₄. To this 0.5 ml of 1N Folin-ciocalteau reagent was added and allowed to stand for 30 min till the blue colour developed correctly. The absorbance (OD) was read within 10 min spectrophotometrically at 600 nm. The specific enzyme activity was expressed as mg tyrosine/h/mg protein using linear regression formula: X = 205.5 Y - 9.8

Invertase Activity:
Invertase activity was determined following the modified method based on that of Hatch and
Glasziou (1963) using the standard curve of glucose and sucrose as a substrate.

To 1 ml enzyme aliquot (illustrated in flow chart I) 1 ml 0.1M sucrose in citrate buffer, (pH 5.4; 0.1M) and 1 ml citrate buffer (pH 5.4; 0.1M) were added and kept at 30°C for 60 minute incubation. To the above 2 ml hot absolut alcohol and 2 ml 5% Na$_2$SO$_4$ were added and boiled, till all the absolute alcohol evaporated. Again volume was made to 5 ml with DW and centrifuged for 15 minute. From the above supernatant 1 ml was added with 1 ml Nelson Somogyi reagent and boiled at 100°C for 15 min, cooled rapidly and to it 1 ml of arsénomolybdate was added and final volume was made upto 20 ml and OD was taken at 540 nm. The invertase activity was calculated and expressed as mg glucose produce/g fresh weight/h using regression formula: $X = 0.00375 \cdot Y$

**Peroxidase:**

The peroxidase activity was determined following the method of George (1953) using guaiacol (as an hydrogen donor) and H$_2$O$_2$ (as a substrate). A 1.0 ml aliquot was mixed with 1.0 ml of phosphate (P$_4$O$_7$) buffer (0.2M, pH 6.4) and 1.0 ml of 20 mM guaiacol (0.22 ml of guaiacol/100 ml). The OD was read at 420 nm. To the same reaction mixture 0.02 ml of H$_2$O$_2$ (20 vol.).

**Soluble enzyme protein:**

The water soluble protein were estimated following the method of Lowry et al. (1951) (slightly modified) for the calculation of specific activities of enzymes. A suitable initial aliquot (0.5 ml) as illustrated in flow chart I was mixed with 4.0 ml of 12.5% (w/v) Na$_2$CO$_3$ and 1.0 ml of 0.1% (w/v) CuSO$_4$. The mixture was allowed to react for 30 min at room temperature. After that, 0.5 ml of 1N folin-ciocalteau reagent was added and the optical density (OD) of the resulting colour was spectrophotometrically read at 600 nm within 10 min. The amount of enzyme protein ($x \mu$g/g fresh weight) was calculated using the following linear regression formula: $X = 655.8 \cdot Y - 9.9$

b. **Metabolic Contents:**

ii) **Preparation of Metabolites Homogenate:** As illustrated in flow chart II.
Embryonic axis and cotyledon weighed separately and homogenised in 10 ml 80% alcohol

| Boiled at 100°C, 2-3 min. then kept overnight in fridge |

| Thrice extracted in 5 ml 80% alcohol, 30°C |

- **Centrifuged**
  - 1) Supernatant I, II, III for reducing, non-reducing sugars by (Wharton, McCarty method, 1972)
  - Residue dissolved in 10 ml DW and boiled at 100°C for 20 min.
  - Cool it

  - **Centrifuged**
    - Supernatant used for starch estimation by (Paleg et al., 1962)
    - Residue dissolved in 5 ml NaOH 30°C, for 30 min.

    - **Centrifuged**
      - Supernatant 5 ml used for protein estimation by (Lowry et al., 1951, slightly modified)
      - Residue (discarded)
Reducing and Non-reducing sugar:

Weighed material was boiled in 80 percent alcohol for 4-5 minute homogenised using a pinch of sand and centrifuged. Residue was again extracted with 5 ml of alcohol twice. The supernatant was taken and total volume was made to 20 ml with distilled water from 20 ml extract 10 ml each was taken for reducing sugar and non-reducing sugars. To extract total sugars 3 ml of 1N HCl (8.75 ml of con. HCl+91.25 ml DW) was added to hydrolyse non-reducing sugars and it was kept in boiling water bath for 20 min. It was cooled and neutralized by adding 3 ml of 1N NaOH. A 1 ml of 25 percent lead acetate and 1 ml of 25% sodium carbonate was added in both the sets and volume was made to 20 ml, then it was filtered. 1 ml of aliquot was taken for sugar estimation by the method of Nelson Somogyi (Wharton and McCarty, 1972).

1 ml of aliquot was mixed with 1 ml Nelson Somogyi reagent. Tubes were capped with glass marbles and heated on waterbath at 100°C for 20 minute and cooled rapidly. 1 ml of arsenomolybdate reagent was added and test tubes frequently shaken for 5 min to dissolve the red precipitate. Final volume was made to 20 ml. OD was taken at 540 nm on spectrophotometer.

Amount of reducing and non-reducing sugar were calculated and expressed in term of mg/g fresh weight, using a regression formula: $X = 297.36 \ Y - 2.56$

Protein:

Estimated by the method of Lowry et al (1951) slightly modified. The residue after removal of sugar was dissolved in 5 ml of 1N NaOH for 20-30 min with intermittent shaking and then centrifuged. From the supernatant, 1 ml of aliquot was taken and to this 4 ml of 12.5 percent Na$_2$CO$_3$ and 1 ml of 0.1 percent CuSO$_4$ was added and incubated at room temperature for 30 minute. To the above 0.5 ml of 1N folin-ciocalteau reagent was added and after 30 minute OD was taken at 600 nm on spectrophotometer. The amount of protein was calculated and expressed as mg/g fresh weight. Regression formula for calculation is: $X = 655.8 \ Y - 9.9$

Starch:

Estimated following the method of Paleg et al. (1962). The residue left after sugar removal was dissolved in 10 ml distilled water (DW) and boiled at 100°C for 20 minute, then it was
cooled and centrifuged and supernatant was collected as starch aliquot. In reaction 1 ml of aliquot, 1 ml citrate buffer pH 5.0 (0.2 M) and 1 ml I₂KI solution was added and incubated at 30°C for 5 minute. The final volume was made up 10 ml with DW and OD was measured at 600 nm on spectrophotometer. The amount of starch was calculated and expressed as mg/g fresh weight using regression formula: X = 0.000655 Y - 0.0039
RESULTS AND DISCUSSION

2.A Standardization Studies:

i) Seed soaking volume, duration and temperature:

The seeds of mungbean, urdbean, cowpea and pigeonpea were soaked in different volumes for different soaking hours (h) at three different temperature regimes, to optimize effective soaking conditions for each type of seed.

The results in Fig. 2.1 to 2.6 showed that effective soaking volume (g/ml) for mungbean was 1:2 for 4 h duration, urdbean 1:2 for 5 h, cowpea 1:6 for 4 h and for pigeonpea 1:6 for 6 h. These soaking conditions in each crop showed improved germination and significant increase in root-shoot length, fresh and dry weight of seedlings. In all the four crops there was no significant increase in root dry weight due to seed soaking, although marginal increase was observed at 4 to 5 h soaking in mungbean and urdbean. Significant increase of shoot and leaf fresh and dry weight at above soaking conditions was observed in all the four crops.

Reduced or excess water supply at less than 2 h and more than 6 h soaking showed reduced germination and inhibited seedling growth in all the four crops. The early stage of water uptake is a crucial phase for the seed, sensitivity to rapid imbibition, chilling, and anoxia may affect seedling establishment and later yield (Obendorf and Hobbs, 1970). Similar results were also obtained by Choudhuri and Choudhuri (1987) regarding presoaking of jute seeds for 3 to 6 h followed by drying which showed least damaging effect on germination and seedling growth.

The period of imbibition by seeds is also a period of sensitivity to temperatures (Pollock, 1969; Leopold, 1980). Our studies show that seed soaking treatments at ambient temperature (26±2°C in BOD) have better seed germination and seedling performance in comparison to soaking at high temperature (i.e. 30-35°C) and low (7°C) temperature in all the four type of seeds. Seed soaking at higher temperatures may be causing rapid imbibition which in turn causes soaking injury and consequently motivating thermal damage within seed (Givelberg et al, 1984). Low temperature may decrease the ability of the tissue components to withstand expansion during the wetting as noted by Willing and Leopold (1983) or it may also make
Fig. 2.1 Germination (%) and seedling performance of mungbean and urdbean seeds soaked in distilled water in different volume (w/v, g/ml)
Fig. 2.2  Germination (%) and seedling performance of cowpea and pigeonpea seeds soaked in distilled water in different volume (w/v, g/ml)
Fig. 2.3 Germination (%) and seedling performance of mungbean and urdbean seeds soaked in distilled water for different durations in hours (h)

**Mungbean**

![Graph of Mungbean Germination and Length](image1.png)

**Urdbean**

![Graph of Urdbean Germination and Length](image2.png)

![Graph of FW (mg) (0)](image3.png)

![Graph of FW (mg) (LAR)](image4.png)

![Graph of dW (mg) (0)](image5.png)

![Graph of dW (mg) (LAR)](image6.png)
Fig. 2.4 Germination (%) and seedling performance of cowpea and pigeonpea seeds soaked in distilled water for different durations in hour (h)

**Cowpea**

![Cowpea Germination and Length](image)

**Pigeonpea**

![Pigeonpea Germination and Length](image)
Fig. 2.5 Germination (%) and seedling performance of mungbean and urdbean seeds soaked in distilled water at different temperatures

**Mungbean**

- Germination (%)
- Length (cm)

**Urdbean**

- Germination (%)
- Length (cm)

Comparison of germination and seedling performance for mungbean and urdbean seeds soaked in distilled water at different temperatures.
Fig. 2.6 Germination (%) and seedling performance of cowpea and pigeonpea seeds soaked in distilled water at different temperatures.

**Cowpea**

- Germination (%)
- Length (cm)

**Pigeonpea**

- FW (mg) (Ø)
- FW (mg) (LAR)

- FW (mg) (Ø)
- FW (mg) (LAR)
embryonic tissue brittle and hence make them susceptible to the chilling injury. Thus the findings suggest that soaking at moderate temperature is the only protection provided against chilling injury and thermal inactivation.

It was evident from the results that among the four crops, seeds of cowpea and mungbean absorbed water more rapidly than urdbean and pigeonpea during early phase of soaking. High temperature and prolonged (>6 h) soaking was found to be deleterious for all type of seeds. Seeds of cowpea and pigeonpea required more water, this may be due to their large surface area in contact with water during imbibition. It is clear here that seed soaking is beneficial only when solution is just sufficient and for shorter duration. Secondly seed soaking treatments need standardization in each seedtype, if beneficial results are to be obtained.

ii) PGR Concentrations:

In this study various plant growth regulators (PGRs) viz. GA$_3$, KIN, NAA, ETH, IBA and ABA were applied in a wide range of concentrations ($10^{-4}$M to $10^{-7}$M) to assess their effectiveness using distilled water as soaking medium. The seeds were soaked in an aqueous solution containing PGRs at standard soaking conditions.

Results in Fig. 2.7 to Fig. 2.10 showed that application of higher dosage of chemicals were deleterious for the germination and seedling growth of all pretreated seeds. Even simple water soaking showed increased seed germination and seedling growth (length, fresh weight, dry weight) than control seeds. Similar results were obtained in seeds of eggplant and radish by hydration - dehydration treatment (Basu and Pal, 1980; Rudrapal and Nakamura, 1988). Furthermore, PGRs at very low concentration (GA$_3$ 10$^{-5}$M and KIN, ETH, NAA at 10$^{-6}$M) showed significant increment in percent germination and seedling length, fresh and dry weight in comparison to untreated (UN) seeds in all the four crops. ABA at its all higher concentration except 10$^{-7}$M showed reduced germination and seedling performance in all crops, similar results were obtained in Sinapis alba (Schopfer et al, 1979). Results of IBA and NAA treatment showed only marginal improvement of seedling performance in comparison to other treatments. Whereas treatment of GA, KIN, ETH, showed significant increase in percent germination, seedling growth and drymatter accumulation, over control seeds. Persson (1988) also
Fig. 2.7: Germination (%) and seedling performance of Mungbean seeds soaked in different concentration of various PGRs.

- Germination (%)
- Shoot Length
- Root Length
- Leaf

Concentration in Mole (M)

- $10^{-4} \text{M} = A$
- $10^{-5} \text{M} = B$
- $10^{-6} \text{M} = C$
- $10^{-7} \text{M} = D$
Fig. 2.8: Germination (%) and seedling performance of Urdbean seeds soaked in different concentration of various PGRs.

- △ Shoot
- ○ Leaf
- ▼ Root

Concentration in Mole (M): $10^{-4} \text{M} = A$, $10^{-6} \text{M} = C$, $10^{-5} \text{M} = B$, $10^{-7} \text{M} = D$
Fig. 2.9: Germination (%) and seedling performance of Cowpea seeds soaked in different concentration of various PGRs.
Fig. 2.10: Germination (%) and seedling performance of Pigeonpea seeds soaked in different concentration of various PGRs.

- Germination (%)
- Shoot Length
- Root Length

Concentration in Mole (M)

\[ 10^{-4} \text{m} = A \]
\[ 10^{-5} \text{m} = B \]
\[ 10^{-6} \text{m} = C \]
\[ 10^{-7} \text{m} = D \]
Fig. 2.11 Germination (%) and seedling performance of mungbean seeds soaked in distilled water and PGRs germinated with drying and without drying
Fig. 2.12 Germination (%) and seedling performance of urdbean seeds soaked in distilled water and PGRs and germinated with drying and without drying.
Fig. 2.13 Germination (%) and seedling performance of cowpea seeds soaked in distilled water and PGRs and germinated with drying and without drying
Fig. 2.14 Germination (%) and seedling performance of pigeonpea seeds soaked in distilled water and PGRs and germinated with drying and without drying

**With Drying**

**Without Drying**
observed higher vigour of many crop seeds with GA, KIN and ETH treatment alone and in combination.

(iii) Fig. 2.11 to 2.14 showed better seed germination and seedling performance of seeds used after slow drying at low temperature in comparison to without drying of seed after soaking in each type of crop.

Only a particular concentration of hormone was found to be effective in all four crops, similar finding was reported in other crops (Basu and Dasgupta, 1974; Basu et al., 1975; Basu, 1976).

Tilden (1985) observed that slow hydration followed by dehydration with PGRs solution produced better seedling and higher germination than UN seeds. This may be due to increase in imbibition and acceleration of germination of seed by interaction of exogenously applied PGRs with endogenous hormones, which in turn may enhance many biochemical (enzyme activity and metabolic) changes in the seed, leading to improved seed germination and seedling growth.

2.B BIOCHEMICAL STUDIES:

i) Enzyme Activities:

In the present study changes in the enzymatic activities of PGRs pretreated seeds of mungbean, urdbean, cowpea and pigeonpea were studied during different hours of germination in the seedling axis and cotyledon separately.

Total Amylase (Fig.2.15, 2.16):

There was significant increase in the activity in the axis of all the four crops in comparison to the activities in the cotyledon. In axis, the initial activity increased at high rate upto 48 h of germination but at later hours rate of increase in activity was very slow. The treatment with GA, KIN, ETH and NAA showed increased amylase activity in their axis in comparison to other treatments and control. ABA treatments showed reduced activity in both the seedling axis and cotyledon whereas IBA showed reduction only in cotyledonary parts during early hours of germination. In cotyledon hydrolysis of starch at initial hours (24) was high but during later hours gradual decrease was observed, this may be due to limited reservoir in cotyledon.
Fig. 2.15 Amylase activity (mg starch hydrolysed/30 min/g fresh weight) of PGRs pretreated mungbean and urdbean seed at different hours of germination.
Fig. 2.16 Amylase activity (mg starch hydrolysed/30 min/g fresh weight) of PGRs pretreated cowpea and pigeonpea seed at different hours (h) of germination.

Cowpea (Axis)  
Cowpea (Cotyledon)  
Pigeonpea (Axis)  
Pigeonpea (Cotyledon)
The increase in activity may be leading to more supply of nutrients to the developing embryo making them to survive effectively under normal conditions. During initial hours increase in hydration increases the internal pressure and primarily reactivates various enzyme synthesis, which in turn set up various physiological manifestation, which results in increased and earlier germination (Woodstock and Grabe, 1967; Bewley and Black, 1982).

Soaking-drying alone significantly increased the amylolytic activity of seed than seed without any soaking treatment (Rudrapal and Basu, 1979). Similar results were obtained here by PGRs seed-soaking treatment. The stimulation of α-amylase expression in barley aleurone layer by GA₃ and the inhibition of this effect by ABA have been well documented (Nolan and Hot, 1988). Petruzzelli and Taranto (1990) found that GA treatment promoted amylase activity in different wheat stocks. Number of other workers have shown the increased amylase activity by GA (Muthukrishnan et al, 1983; Mac Gregor et al., 1984; Fincher, 1989; Sopanen and Lurierere, 1989; Pandya, 1992).

Afifi et al (1986) reported decreased activity of α-amylase and peroxidase during wheat germination. Ashford and Gubler (1984) gave a possible explanation that the reduced amylase activity is due to a deficiency of gibberellins and other unknown factors released. Higher level of inhibitory substances in extra embryonic tissues may be responsible for the loss of activity (Petruzzelli and Taranto, 1989). This data corroborates the earlier finding that GA₃ induce α-amylase in cotyledon of germinating pea (Locker and Ilan, 1975, besides this kinetin was also found to be promoting α-amylase activity in bean cotyledon (Gepstain and Ilan, 1970).

Protease (Fig. 2.17, 2.18):

The activity showed indefinite trend in the embryonic axis, first showing decrease upto 72 h of germination but later at 96 h it showed increase in almost all type of treatments including control. KIN, GA and ETH treatment showed slightly higher protease activity in axis in comparison to other treatments and control. The trend of this activity is almost similar in all
Fig. 2.17 Protease activity (mg tyrosine released/mg protein) of PGRs pretreated mungbean and urdbean seed at different hours (h) of germination.
Fig. 2.18 Protease activity (mg tyrosine released/mg protein) of PGRs pretreated cowpea and pigeonpea seed at different hours (h) of germination.
the four type of crop in both, axis and cotyledon during germination. At 24h, the activity in cotyledons was very low but as germination progressed the activity increased significantly till 72 h and then once again sharp drop was observed in the activity at 96 h of germination. Here the increase in the activity through pretreatment showed the order KIN > ETH > GA > DW > NAA lastly in control. The ABA and IBA treatment exhibited reduction in protease activity. In urdbean and pigeonpea the results of GA3 pretreatment showed increased activity in comparison to KIN treatment but in mungbean and cowpea results were vice versa.

Perl et al (1978) demonstrated that the increase in proteolytic activity could affect all other enzyme levels because proteolytic enzymes play an important role in the seed germination process showing the quantitative inverse correlation between protein synthesis and proteolytic activity on one hand and release of ample amounts of ninhydrin reactive compound on the other hand. Here initial activity was more to release more aminoacid (the pioneer compound for protein synthesis) so, that required protein demands for axis growth can be fulfilled but as soon as axis germinated to an extent the limitation in the breakdown of protein takes place. The levels of these enzymes is under regulation of PGRs. These findings are in close agreement with the other observation (Ryan, 1973; Mehra, 1990; Pandya, 1992).

An increased protease activity may be the cause of the declination in other enzymes (Perl et al, 1978). Studies on different crops have shown similar patterns from our laboratory (Saxena, 1979; Pakeeraiah, 1985; Pandey, 1987; Mehra, 1990; Pandya, 1992). Protease, amylase and peroxidase was stimulated by GA application and reduced by ABA application (Bhatia and Saxena, 1990; Harvey and Oaks, 1974). KIN increased protease activity in squash cotyledons (Penner and Ashton, 1966). These findings are in support of the finding that proper activation of hydrolytic enzymes activate seed germination and seedling performance (Singh, 1984; Saxena, 1988).

Invertase (Fig. 2.21, 2.22): The invertase activity was very less in all four types of pulse crops during germination. Observation in embryo axis showed that seeds treated with KIN, ETH and GA had slight increase
Fig. 2.21 **Invertase activity** (mg reducing sugar released/hr/g fresh weight) of PGRs pretreated mungbean and urdbean seed at different hours (h) of germination.

**Mungbean (Axis)**

**Mungbean (Cotyledon)**

**Urdbean (Axis)**

**Urdbean (Cotyledon)**
Fig. 2.22 Invertase activity (mg reducing sugar released/hr/g fresh weight) of PGRs pretreated cowpea and pigeonpea seed at different hours (h) of germination.

Cowpea (Axis)

Cowpea (Cotyledon)

Pigeonpea (Axis)

Pigeonpea (Cotyledon)
in activity in comparison to other treatments and UN seed. The result of ABA and IBA treatments showed reduced activity. The overall trend showed gradual increase up to 72 h then decrease at 96 h in axis where the activity was slightly more than in cotyledon. In cotyledon only marginal increase was observed in activity till 96 h of germination. In urdbean and pigeonpea cotyledons, GA pretreatment increased the activity in comparison to ETH and KIN but in mungbean and cowpea, KIN and ETH treatment shows increased invertase activity. Stimulation of peroxidase, invertase and amylase activity by pretreatment was also reported by Singh and Saxena (1990). Heydecker et al (1974) also showed that during pretreatment, all processes requisite of germination are completed. The PGRs treatment in few systems, known to trigger the release of several enzymes during the germination.

**Peroxidase (Fig. 2.19, 2.20):**

The direct expression of optical density showed that peroxidase activity was significantly increased in all the four crops through various PGR pretreatments. Axis showed more increment in activity in comparison to cotyledon. The axis in all type of treatment shows increase up to 96 h but cotyledon in turn shows decrease after 72 h of germination. The results of KIN, ETH, GA and NAA treatment showed more increase in activity in comparison to other treatments and control in the axis, but in cotyledon GA treatment shows more increase in activity than other treatments. Cowpea and pigeonpea embryo axis have significantly high activity whereas urdbean cotyledon has significantly low activity in all type of treatments. Thus the rate of change of peroxidase activity in pulses crop was found to be slower.

Peroxidases are enzymes which fulfill many function in plant cell wall: biosynthesis of hydrogen peroxide, polymerisation of lignin (Gaspar et al, 1982). Basra et al (1989) observed an increase in peroxidase activity with GA and ABA. In contrary, Fry (1979, 1980) found suppression of peroxidase activity with GA by inhibiting the secretion of peroxidase, who proposed that GA exerted its action through a cellular restribution of calcium, which modified peroxidase secretion. Other positive effects of GA treatment on peroxidase activity in various crops during germination were also reported (Bhatia and Saxena, 1990; Pandya, 1992). Kinetin treatment also showed increased peroxidase activity (Gasper et al, 1973; Sharma et al, 1977).
Fig. 2.19 Peroxidase activity (O.D./10 min/gm fresh weight) of PGRs pretreated mungbean and urdbean seed at different hours (h) of germination.

Mungbean (Axis)

Mungbean (Cotyledon)

Urdbean (Axis)

Urdbean (Cotyledon)
Fig. 2.20 Peroxidase activity (O.D./10 min/gm fresh weight) of PGRs pretreated cowpea and pigeonpea seed at different hours (h) of germination.

Cowpea (Axis)

Cowpea (Cotyledon)

Pigeonpea (Axis)

Pigeonpea (Cotyledon)
Reduced activity was observed by ABA application (Fries, 1972; Gasper et al., 1973).

The treatment with ETH seem to regulate peroxidase activity at some step following the transcription. Sachar and Berry (1984) found that hormone treatment could enhance the preferential synthesis of gene transcripts, eventually resulting in the enzyme induction peroxidase have been considered as a scavenger (Fridovich, 1976) which nullifies the harmful effects of metabolic intermediates and endproducts.

Higher peroxidase activity is indicative of higher plant potential (Sircar and Choudhary, 1980; Bhattacharjee and Gupta, 1981; 1984). In this study, the results were correlated with the seedling performance and germinability which shows strong positive relationship between peroxidase activity and the germinability.

**Enzyme Protein (Fig. 2.23, 2.24):**

The soluble enzyme protein showed indefinite trend in the embryo axis. There was increasing trend up to 48 h then activity decreased at 72 h and once again it increased at 96 h of germination in almost all the four crops and in each type of treatment. GA and KIN treatments showed significant increased than other type of treatments and control. There was marked increase in soluble enzyme protein also in the cotyledonary part during the early hours of germination but at 96 h once again there was decrease in activity. The more amount of enzyme protein in the cotyledon was in KIN > ETH > GA than in other treatments like NAA and DW. ABA and IBA shows reduction in enzyme protein of the cotyledon of all crops, except in urdbean. More amount of soluble enzyme protein was found in pigeonpea > cowpea > mungbean and lastly in urdbean.

Barendse (1983) suggested that, hormones, apart from influencing the synthesis of enzyme proteins, directly or indirectly may also influence the activity of both synthetic and degradative enzymes. It also play a major role in modifying the conformation of enzyme proteins, e.g. RNA polymerase. Auxin, ethylene and GA₃ differently activated growth and activity of peroxidase in mungbean seedling (Dendsey and Sachar, 1982).

It is clear from these studies that pretreatment with various PGRs to seeds of pulse crop enhance some of the hydrolytic enzymatic activities with different levels at different hours of
Fig. 2.23 Enzyme Protein content (mg/g fresh weight) of PGRs pretreated mungbean and urdbean seed at different hours (h) of germination.
Fig. 2.24 **Enzyme Protein content** (mg/g fresh weight) of PGRs pretreated cowpea and pigeonpea seed at different hours (h) of germination.
germination, causing early and improved seed germination and seedling growth.

ii) Metabolic Contents:

Due to very low water content in air-dried seeds metabolic activity is practically at a stand
still (Street, 1966) with imbibition the moisture level of seed increases and this results in rise
of various metabolic activity in seed during germination. In the present study changes in vari-
ous metabolic content in seedling axis and cotyledon were carried out at different germination
hours in PGRs pretreated seeds.

Reducing Sugar (Fig. 2.25, 2.26):

Reducing sugar content in axis increased from 24 h to 96 h significantly in all the four crop
whereas in cotyledon it showed a fluctuating trend, increased content from 24 h to 72 h and
then it decreased at 96 h, showing utilization and biosynthesis simultaneously and there was
higher sugar production in GA, KIN, ETH treatment in comparison to other treatment and
control in both axis and cotyledon. ABA and IBA showed reduced sugar content in comparison
to control.

Non-Reducing Sugars (Fig. 2.27, 2.28):

In embryo axis non-reducing sugar content was highly fluctuating. It showed increase till 72 h
and then decrease at 96 h of germination. Whereas in cotyledon it showed decreasing trend
upto 48 h, then it increased at 72 h and again decreased at 96 h of germination in almost all
four type of crop. KIN, GA, ETH and NAA treatment showed increase in non-reducing sugar
content in comparison to UN. IBA and ABA exhibited significant reduction in sugar content of
cotyledon except in mungbean.

Overall in axis reducing sugar content increased mani-fold during germination, in contrast to
that in cotyledon in all type of treatments. Briggs (1968) showed that imbibition of water helps
in releasing sugar during germination. It has been already reported that α-amylase activity is
enhanced by various PGRs consequently reducing sugars are produced from starch at faster
rate, similar findings are reported by many workers (Paleg, 1960, 1965; Yomo, 1961; Varner,
1965) but Sprent (1968) demonstrated contrary report of Paleg's findings.
Fig. 2.25 Reducing Sugar content (mg/g fresh weight) of PGRs pretreated mungbean and urdbean seed at different hours (h) of germination

Mungbean (Axis)  Mungbean (Cotyledon)

Urdbean (Axis)  Urdbean (Cotyledon)
Fig. 2.26 Reducing Sugar content (mg/g fresh weight) of PGRs pretreated cowpea and pigeonpea seed at different hours (h) of germination.

Cowpea (Axis)

Cowpea (Cotyledon)

Pigeonpea (Axis)

Pigeonpea (Cotyledon)
Fig. 2.27 Non-reducing Sugar content (mg/g fresh weight) of PGRs pretreated mungbean and urdbean seed at different hours (h) of germination.

Mungbean (Axis)

Mungbean (Cotyledon)

Urdbean (Axis)

Urdbean (Cotyledon)
Fig. 2.28 Non-reducing Sugar content (mg/g fresh weight) of PGRs pretreated cowpea and pigeonpea seed at different hours (h) of germination.
During germination the entire metabolic apparatus of seed become operative in a controlled, sequential fashion, where PGRs play key role. This could be related to the fact that GA$_3$, KIN and other PGRs pretreatment of seeds may have caused the release of hydrolytic enzymes required for breakdown of stored starch to simple sugar and their translocation to embryo where they serve as respiratory substrate for growth (Shafiq, 1980; Bhardwaj and Masoodi, 1992).

**Total Protein (Fig. 2.29, 2.30):**

In axis protein content significantly increased from 24 h to 72 h and then decreased slightly at 96 h of germination. GA treated seeds showed higher total protein content over other treatment in mungbean and cowpea, whereas KIN and ETH treatment increased protein content in urdbean and pigeonpea crop. Here also ABA and IBA showed reduced protein content in comparison to UN both in axis and cotyledon. The cotyledon had high total soluble protein content initially but there was gradual decline in it as germination proceeded in each type of treatment. Here also KIN, GA, NAA and ETH showed higher protein content in it in comparison to other treatment and control.

Initially observed higher protein content in cotyledon and lower in embryo axis may be due to slow translocation of protein from cotyledon to axis at early hours of germination or due to break down of protein into amino acid as a result of protease activity as mentioned earlier. Significant increase in RNA and protein through various PGRs application was obtained by many workers in different crop (Joshi et al, 1993; Maheswari, 1987; Johri and Varner, 1968). Finding shows that role of PGRs action was sequential in nature, not only in growth but also in terms of macromolecular contents.

**Starch (Fig. 2.31, 2.32):**

Starch content increased in embryonic axis as germination proceed in all four type of crop. Here in axis, KIN, ETH and NAA treatment showed significant increase in starch content than GA, DW and control seeds. The results of GA treatment showed less increment in starch content in both axis and cotyledon, this may be due to higher amylase activity during initial
Fig. 2.29 Protein content (mg/g fresh weight) of PGRs pretreated mungbean and urdbean seed at different hours (h) of germination.
Fig. 2.30 Protein content (mg/g fresh weight) of PGRs pretreated cowpea and pigeonpea seed at different hours (h) of germination.

Cowpea (Axis)

Cowpea (Cotyledon)

Pigeonpea (Axis)

Pigeonpea (Cotyledon)
Fig. 2.31 Starch content (mg/g fresh weight) of PGRs pretreated mungbean and urdbean seed at different hours (h) of germination.
Fig. 2.32 Starch content (mg/g fresh weight) of PGRs-pretreated cowpea and pigeonpea seed at different hours (h) of germination.

Cowpea (Axis)

Cowpea (Cotyledon)

Pigeonpea (Axis)

Urdbeana (Cotyledon)
germination as mentioned earlier. In cotyledon, treated with IBA and ABA, more starch accumulation was obtained in all the four type of crop in comparison to other treatments and control, this may be due to their inhibitory action on amylase activity (Chrispeels and Varner, 1966; Roy et al, 1973).

Thus sequence of biochemical events during germination results from sequential appearance of different enzyme activities, that compiles with the need of germinating seed and allow mobilization of stored material from cotyledon to embryo axis for proper growth and development.
### Table 2.1: Analyses of Variance

(a) Enzyme Activities data of PGRs pretreated seeds

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* *, **: Significant at 5 and 1 percent level, respectively.
CONCLUSIONS:
Incorporation of various PGRs in seed through presoaking is well documented through various controversial views but its practical applications are still in their infancy.
Among controversial literature related to presoaking treatment, pretreatment requires more attention to the methods of application, time to exposure and uniformity of application along with proper dosages. Our findings suggested four hour soaking in double the volume is better for mungbean, for urdbean five hour in double the volume, cowpea four hours in six time volume and for pigeonpea six hours in six time volume of soaking solution are beneficial for germination and seedling performance. Moderate temperature 26±2°C was found to be most effective for all the types of seed treatments. This optimum volume, duration and temperature during soaking prevented various soaking injuries in seeds.
The better seedling performance was observed through application of effective dosages of particular PGRs. Here it ranges between 10⁻⁵M to 10⁻⁷M concentration of each PGRs for all four crop seeds.
During imbibition (germination) the proteneous seed of pulses become hydrated due to their colloids and several hydrolytic enzymes like amylase, protease, invertase regain their activity, where various incorporated PGRs trigger the mobilization of various metabolites from cotyledon to the growing embryonic axis. GA, KIN, NAA and ETH pretreatment were effective than other treatments and control during all the stages of seed germination. They activate better seed germination and seedling performance by effective physiological and biochemical manipulation during seed germination and early seedling growth.